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Terms	Documents
L4 and deacylas$4	4

Database:

US Pre-Grant Publication Full-Text Database
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IBM Technical Disclosure Bulletins

Search: L5

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Search History

DATE: Wednesday, June 16, 2004 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
	DB=PGPB,USPT,EPAB,JPAB,DWPI; PLUR=YES; OP=OR		
L5	L4 and deacylas$4	4	L5
L4	L2 and (steinbuchel or priefert or rabenhorst).in.	22	L4
L3	L2 and deacylas$4	5	L3
L2	(ferul$4 same acid$4) and vanilli$4	352	L2
	DB=USPT; PLUR=YES; OP=OR		
L1	(5712132 or 5510252 or 5358861 or 5128253 or 4847422 or 5017388).pn.	6	L1

END OF SEARCH HISTORY

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Search Results - Record(s) 1 through 5 of 5 returned.

1. Document ID: US 20030228670 A1

Using default format because multiple data bases are involved.

L3: Entry 1 of 5 File: PGPB Dec 11, 2003

PGPUB-DOCUMENT-NUMBER: 20030228670
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030228670 A1

TITLE: Synthetic enzymes for the production of coniferyl alcohol, coniferylaldehyde, ferulic acid, vanillin and vanillic acid and their use

PUBLICATION-DATE: December 11, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Steinbuchel, Alexander	Altenberge		DE	
Priefert, Horst	Telgte		DE	
Rabenhorst, Jurgen	Hoxter		DE	

US-CL-CURRENT: 435/136; 435/147, 435/189, 435/252.3, 435/254.2, 435/320.1, 435/69.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. De

2. Document ID: US 20020182697 A1

L3: Entry 2 of 5 File: PGPB Dec 5, 2002

PGPUB-DOCUMENT-NUMBER: 20020182697
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020182697 A1

TITLE: SYNTHETIC ENZYMES FOR THE PRODUCTION OF CONIFERYL ALCOHOL, CONIFERYLALDEHYDE, FERULIC ACID, VANILLIN AND VANILLIC ACID AND THEIR USE

PUBLICATION-DATE: December 5, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
STEINBUCHEL, ALEXANDER	ALTENBERGE		DE	

PRIEFERT, HORST	TELGTE	DE
RABENHORST, JURGEN	HOXTER	DE

US-CL-CURRENT: 435/189; 435/156, 435/320.1, 435/325, 536/23.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. De

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3. Document ID: US 6524831 B2

L3: Entry 3 of 5 File: USPT Feb 25, 2003

US-PAT-NO: 6524831
DOCUMENT-IDENTIFIER: US 6524831 B2

TITLE: Synthetic enzymes for the production of coniferyl alcohol, coniferylaldehyde, ferulic acid, vanillin and vanillic acid and their use

DATE-ISSUED: February 25, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Steinbuchel; Alexander	Altenberge			DE
Priefert; Horst	Telgte			DE
Rabenhorst; Jurgen	Hoxter			DE

US-CL-CURRENT: 435/156; 435/189, 435/25, 435/252.3, 435/320.1, 435/325, 435/69.1, 536/23.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. De

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4. Document ID: US 6331655 B1

L3: Entry 4 of 5 File: USPT Dec 18, 2001

US-PAT-NO: 6331655
DOCUMENT-IDENTIFIER: US 6331655 B1

TITLE: Process for the preparation of aromatic carbonyl compounds from styrenes

DATE-ISSUED: December 18, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gatfield; Ian-Lucas	Hoxter			DE
Hilmer; Jens-Michael	Hoxter			DE

US-CL-CURRENT: 568/435; 435/147, 560/104, 560/105, 562/495, 562/496, 568/310, 568/311, 568/426

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. De

☐ 5. Document ID: US 20030228670 A1, EP 845532 A2, DE 19649655 A1, JP 10155496 A, US 20020182697 A1, US 6524831 B2

L3: Entry 5 of 5 File: DWPI Dec 11, 2003

DERWENT-ACC-NO: 1998-288749
DERWENT-WEEK: 200382

TITLE: New enzymes of eugenol catabolism, DNA, cosmid clones, vectors and transformed microorganisms - used to synthesise coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin and vanillic acid

INVENTOR: PRIEFERT, H; RABENHORST, J ; STEINBUECHEL, A ; STEINBUCHEL, A

PRIORITY-DATA: 1996DE-1049655 (November 29, 1996)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 20030228670 A1	December 11, 2003		000	C12P007/40
EP 845532 A2	June 3, 1998	G	108	C12N015/53
DE 19649655 A1	June 4, 1998		000	C12N015/53
JP 10155496 A	June 16, 1998		110	C12N015/09
US 20020182697 A1	December 5, 2002		000	C07H021/02
US 6524831 B2	February 25, 2003		000	C12N009/02

INT-CL (IPC): C07 C 59/64; C07 H 21/02; C07 H 21/04; C12 N 1/00; C12 N 1/18; C12 N 1/21; C12 N 9/02; C12 N 9/04; C12 N 9/80; C12 N 9/88; C12 N 15/09; C12 N 15/53; C12 N 15/60; C12 N 15/63; C12 N 15/74; C12 P 7/22; C12 P 7/24; C12 P 7/40; C12 P 7/42; C12 P 21/02; C12 Q 1/26; C12 N 1/21; C12 R 1:19; C12 P 7/40; C12 R 1:19

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw. De

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Terms	Documents
L2 and deacylas$4	5

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(FILE 'HOME' ENTERED AT 16:49:17 ON 16 JUN 2004)

INDEX 'ADISCTI, ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, AQUASCI, BIOBUSINESS, BIOCOMMERCE, BIOSIS, BIOTECHABS, BIOTECHDS, BIOTECHNO, CABA, CANCERLIT, CAPLUS, CEABA-VTB, CEN, CIN, CONFSCI, CROPB, CROPU, DISSABS, DDFB, DDFU, DGENE, DRUGB, DRUGMONOG2, ...' ENTERED AT 16:49:40 ON 16 JUN 2004

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        SEA DEACYLAS? (S) VANILL? (S) FERU?
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        1  FILE BIOTECHABS
        1  FILE BIOTECHDS
        1  FILE CAPLUS
        0* FILE FEDRIP
        3  FILE IFIPAT
        3  FILE USPATFULL
        1  FILE USPAT2
        1  FILE WPIDS
        1  FILE WPINDEX
L1        QUE DEACYLAS? (S) VANILL? (S) FERU?
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        SEA (DEACYLAS?(S)FERU?) AND VANILL?
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        1  FILE BIOTECHABS
        1  FILE BIOTECHDS
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        0* FILE FEDRIP
        3  FILE IFIPAT
        3  FILE USPATFULL
        1  FILE USPAT2
        1  FILE WPIDS
        1  FILE WPINDEX
L2        QUE (DEACYLAS?(S) FERU?) AND VANILL?
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FILE 'IFIPAT, USPATFULL, BIOTECHDS, CAPLUS, USPAT2, WPIDS' ENTERED AT 16:52:16 ON 16 JUN 2004

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L3      10 S (DEACYLAS?(S)FERU?) AND VANILL?
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                  CA/CAplus
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                  changes
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 NEWS  8  MAR 03  FRANCEPAT now available on STN
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 NEWS 11  MAR 29  New monthly current-awareness alert (SDI) frequency in RAPRA
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                  available
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 NEWS 15  APR 27  NLDB: New search and display fields available
 NEWS 16  May 10  PROUSDDR now available on STN
 NEWS 17  May 19  PROUSDDR: One FREE connect hour, per account, in both May
                  and June 2004
 NEWS 18  May 12  EXTEND option available in structure searching
 NEWS 19  May 12  Polymer links for the POLYLINK command completed in REGISTRY
 NEWS 20  May 17  FRFULL now available on STN
 NEWS 21  May 27  STN User Update to be held June 7 and June 8 at the SLA 2004
                  Conference
 NEWS 22  May 27  New UPM (Update Code Maximum) field for more efficient patent
                  SDIs in CAplus
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 NEWS 24  May 27  Explore APOLLIT with free connect time in June 2004

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               MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP),
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=> index bioscience medicine
FILE 'DRUGMONOG' ACCESS NOT AUTHORIZED
COST IN U.S. DOLLARS                              SINCE FILE     TOTAL
                                                       ENTRY   SESSION
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FULL ESTIMATED COST                                      0.21        0.21

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       BIOCOMMERCE, BIOSIS, BIOTECHABS, BIOTECHDS, BIOTECHNO, CABA, CANCERLIT,
       CAPLUS, CEABA-VTB, CEN, CIN, CONFSCI, CROPB, CROPU, DISSABS, DDFB, DDFU,
       DGENE, DRUGB, DRUGMONOG2, ...' ENTERED AT 16:49:40 ON 16 JUN 2004

73 FILES IN THE FILE LIST IN STNINDEX

Enter SET DETAIL ON to see search term postings or to view
search error messages that display as 0* with SET DETAIL OFF.

=> s deacylas? (s) vanill? (s) feru?
          1    FILE BIOTECHABS
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  38 FILES SEARCHED...
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          1    FILE USPAT2
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   8 FILES HAVE ONE OR MORE ANSWERS,   73 FILES SEARCHED IN STNINDEX

L1    QUE DEACYLAS? (S) VANILL? (S) FERU?

=> d rank
F1            3    IFIPAT
F2            3    USPATFULL
F3            1    BIOTECHABS
F4            1    BIOTECHDS
F5            1    CAPLUS
F6            1    USPAT2
F7            1    WPIDS
F8            1    WPINDEX


=> s (deacylas?(s)feru?) and vanill?
          1    FILE BIOTECHABS
          1    FILE BIOTECHDS
          1    FILE CAPLUS
          0*   FILE FEDRIP
          3    FILE IFIPAT
  61 FILES SEARCHED...
          3    FILE USPATFULL
          1    FILE USPAT2
          1    FILE WPIDS
          1    FILE WPINDEX

   8 FILES HAVE ONE OR MORE ANSWERS,   73 FILES SEARCHED IN STNINDEX

L2    QUE (DEACYLAS?(S) FERU?) AND VANILL?

=> d rank
F1            3    IFIPAT
F2            3    USPATFULL
F3            1    BIOTECHABS
F4            1    BIOTECHDS
F5            1    CAPLUS
F6            1    USPAT2
F7            1    WPIDS
F8            1    WPINDEX


=> file f1-f8
COST IN U.S. DOLLARS                               SINCE FILE      TOTAL
                                                        ENTRY    SESSION
FULL ESTIMATED COST                                      2.28       2.49
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FILE 'WPINDEX' ACCESS NOT AUTHORIZED

=> s (deacylas?(s)feru?) and vanill?
L3          10 (DEACYLAS?(S) FERU?) AND VANILL?

=> dup rem l3
PROCESSING COMPLETED FOR L3
L4             6 DUP REM L3 (4 DUPLICATES REMOVED)

=> d ti l4 1-6

L4   ANSWER 1 OF 6  IFIPAT  COPYRIGHT 2004 IFI on STN  DUPLICATE 1
TI    SYNTHETIC ENZYMES FOR THE PRODUCTION OF CONIFERYL ALCOHOL,
      CONIFERYLALDEHYDE, FERULIC ACID, VANILLIN AND VANILLIC
      ACID AND THEIR USE

L4   ANSWER 2 OF 6  IFIPAT  COPYRIGHT 2004 IFI on STN
TI    SYNTHETIC ENZYMES FOR THE PRODUCTION OF CONIFERYL ALCOHOL,
      CONIFERYLALDEHYDE, FERULIC ACID, VANILLIN AND VANILLIC
      ACID AND THEIR USE; FOR PRODUCING CONIFERYL ALCOHOL FROM EUGENOL IN
      PRESENCE OF EUGENOL HYDROXYLASE; PRODUCING CONIFERYLALDEHYDE FROM
      CONIFERYL ALCOHOL IN PRESENCE OF CONIFERYL ALCOHOL DEHYDROGENASE;
      PRODUCING FERULIC ACID FROM CONIFERYLALDEHYDE

L4   ANSWER 3 OF 6  IFIPAT  COPYRIGHT 2004 IFI on STN  DUPLICATE 2
TI    SYNTHETIC ENZYMES FOR THE PRODUCTION OF CONIFERYL ALCOHOL,
      CONIFERYLALDEHYDE, FERULIC ACID, VANILLIN AND VANILLIC
      ACID AND THEIR USE

L4   ANSWER 4 OF 6  USPATFULL on STN
TI     Process for the preparation of aromatic carbonyl compounds from styrenes

L4    ANSWER 5 OF 6  BIOTECHDS COPYRIGHT 2004 THOMSON DERWENT/ISI on STN
TI    New enzyme of eugenol catabolism, DNA, cosmid clones, vectors and
      transformed microorganisms;
         coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin
         and vanillic acid production via enzyme-catalyzed eugenol
         catabolism; cosmid clone and vector plasmid pVK100 expression in
         Pseudomonas sp.

L4   ANSWER 6 OF 6  CAPLUS  COPYRIGHT 2004 ACS on STN
TI   Eugenol-catabolizing enzymes, recombinant microbes expressing these
     enzymes, and production of coniferyl alcohol, coniferyl aldehyde, ferulic
     acid, vanillin, vanillic acid with said enzymes


=> d ibib abs l4 1-6

L4   ANSWER 1 OF 6  IFIPAT  COPYRIGHT 2004 IFI on STN  DUPLICATE 1
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AN                          10484240  IFIPAT;IFIUDB;IFICDB
TITLE:                      SYNTHETIC ENZYMES FOR THE PRODUCTION OF CONIFERYL
                            ALCOHOL, CONIFERYLALDEHYDE, FERULIC ACID,
                            VANILLIN AND VANILLIC ACID AND
                            THEIR USE
INVENTOR(S):                Priefert; Horst, Telgte, DE
                            Rabenhorst; Jurgen, Hoxter, DE
                            Steinbuchel; Alexander, Altenberge, DE
PATENT ASSIGNEE(S):         Unassigned
AGENT:                      NORRIS McLAUGHLIN & MARCUS, P.A., 30TH FLOOR, 220
                            EAST 42ND STREET, NEW YORK, NY, 10017, US

                               NUMBER            PK    DATE
                            -----------------    --  --------
PATENT INFORMATION:         US 2003228670        A1  20031211
APPLICATION INFORMATION:    US 2000-750986           20001228

                                                            GRANTED PATENT NO.
                            APPLN. NUMBER              DATE    OR STATUS
                            -----------------        --------  ------------------
DIVISION OF:                US 1997-976063           19971121  6524831

                               NUMBER                  DATE
                            -----------------        --------
PRIORITY APPLN. INFO.:      DE 1996-196496551        19961129
FAMILY INFORMATION:         US 2003228670            20031211
                            US 6524831
DOCUMENT TYPE:              Utility
                            Patent Application - First Publication
FILE SEGMENT:               CHEMICAL
                            APPLICATION
NUMBER OF CLAIMS:           13
AB     The present invention relates to synthetic enzymes for the production of
       coniferyl alcohol, coniferylaldehyde, ferulic acid, vanillin
       and vanillic acid, the use thereof for the production of
       coniferyl alcohol, coniferylaldehyde, ferulic acid, vanillin
       and vanillic acid, DNA coding for these enzymes and
       microorganisms transformed with this DNA.
CLMN   13

L4    ANSWER 2 OF 6   IFIPAT  COPYRIGHT 2004 IFI on STN
AN                          03834742  IFIPAT;IFIUDB;IFICDB
TITLE:                      SYNTHETIC ENZYMES FOR THE PRODUCTION OF CONIFERYL
                            ALCOHOL, CONIFERYLALDEHYDE, FERULIC ACID,
                            VANILLIN AND VANILLIC ACID AND
                            THEIR USE; FOR PRODUCING CONIFERYL ALCOHOL FROM
                            EUGENOL IN PRESENCE OF EUGENOL HYDROXYLASE; PRODUCING
                            CONIFERYLALDEHYDE FROM CONIFERYL ALCOHOL IN PRESENCE
                            OF CONIFERYL ALCOHOL DEHYDROGENASE; PRODUCING FERULIC
                            ACID FROM CONIFERYLALDEHYDE
INVENTOR(S):                Priefert; Horst, Telgte, DE
                            Rabenhorst; Jurgen, Hoxter, DE
                            Steinbuchel; Alexander, Altenberge, DE
PATENT ASSIGNEE(S):         Haarmann & Reimer GmbH, Holzminden, DE
PRIMARY EXAMINER:           Prouty, Rebecca E
ASSISTANT EXAMINER:         Ramirez, Delia
AGENT:                      Norris McLaughlin & Marcus

                               NUMBER            PK    DATE
                            -----------------    --  --------
PATENT INFORMATION:         US 6524831           B2  20030225
                            US 2002182697        A1  20021205
APPLICATION INFORMATION:    US 1997-976063           19971121
EXPIRATION DATE:            21 Nov 2017

                               NUMBER                  DATE
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PRIORITY APPLN. INFO.:      DE 1996-19649655         19961129
FAMILY INFORMATION:         US 6524831               20030225
                            US 2002182697          20021205
DOCUMENT TYPE:              Utility
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FILE SEGMENT: CHEMICAL
GRANTED
NOTE: INDEXED FROM APPLICATION
MICROFILM REEL NO: 008830 FRAME NO: 0814
NUMBER OF CLAIMS: 7
GRAPHICS INFORMATION: 1 Drawing Sheet(s), 1 Figure(s).
DESCRIPTION OF FIGURES:

FIG. 1 is a physical map of cloned fragments, subfragments, and sequenced areas.

The present invention relates to synthetic enzymes for the production of coniferyl alcohol, coniferylaldehyde, **ferulic** acid, **vanillin** and **vanillic** acid from eugenol.

Synthetic enzymes according to the invention are for example:

a) eugenol hydroxylase,
b) coniferyl alcohol dehydrogenase,
c) coniferylaldehyde dehydrogenase,
d) **ferulic** acid **deacylase** and
e) **vanillin** dehydrogenase.

The invention also relates to DNA coding for the abovementioned enzymes and cosmid clones containing this DNA as well as vectors containing this DNA and microorganisms transformed with the DNA or the vectors. It also relates to the use of the DNA for the transformation of microorganisms for the production of coniferyl alcohol, coniferylaldehyde, **ferulic** acid, **vanillin** and **vanillic** acid. The invention also relates to partial sequences of the DNA and functional equivalents. Functional equivalents are understood to be those derivatives in which individual nucleobases have been substituted (wobble substitutions) without resulting in any functional changes. In relation to proteins, amino acids can also be substituted without resulting in any functional changes.

The invention also relates to the individual steps for the production of coniferyl alcohol, coniferylaldehyde, **ferulic** acid, **vanillin** and **vanillic** acid from eugenol, i.e. in concrete terms:

a) the process for the production of coniferyl alcohol from eugenol carried out in the presence of eugenol hydroxylase;
b) the process for the production of coniferylaldehyde from coniferyl alcohol carried out in the presence of coniferyl alcohol dehydrogenase;
c) the process for the production of **ferulic** acid from coniferylaldehyde carried out in the presence of coniferylaldehyde dehydrogenase;
d) the process for the production of **vanillin** from **ferulic** acid carried out in the presence of **ferulic** acid **deacylase**;
e) the process for the production of **vanillic** acid from ***vanillin*** carried out in the presence of **vanillin** dehydrogenase.

After NMG mutagenesis mutants with defects in individual stages of the catabolism of eugenol were obtained from the eugenolutilising Pseudomonas sp. strain HR 199 (DSM 7063). Using total DNA of wild-type Pseudomonas sp. HR 199 partially digested with EcoRI a gene library was constructed in the pVK100 cosmid, which has a broad host spectrum and can also be replicated in stable form in pseudomonads. After packaging in 1-phage particles the hybrid cosmids were transduced to E. coli S17-1. The gene library comprised 1330 recombinant E. coli S17-1 clones. The hybrid cosmid of each clone was transferred by conjugation into two eugenol-negative mutants (mutants 6164 and 6165) of the Pseudomonas sp. HR 199 strain and tested for a possible capacity for complementation. In this test two hybrid cosmids (pE207 and pE115) were identified, the obtainment of which restored mutant 6165's capacity to utilise eugenol. One hybrid cosmid (pE5-1) resulted in the complementation of mutant 6164.

The complementing capacity of plasmids pE207 and pE115 was attributed to a 23 kbp EcoRI fragment (E230). A physical map of this fragment was prepared and the fragment completely sequenced. The genes vanA and vanB which code for ***vanillate*** demethylase were localised in a 11.2 kbp HindIII subfragment (H110).

Another open reading frame (ORF) was found to be homologous to gglutamyl cysteine synthetase produced by Escherichia coli. An additional ORF, which was homologous to formaldehyde dehydrogenases, was identified between the aforementioned ORF and the vanB gene. Two additional ORF's were found to be homologous to the cytochrome C subunit or the flavoprotein subunit of p-cresol methylhydroxylase, respectively produced by Pseudomonas putida. In the Pseudomonas sp. HR 199 strain, these ORF's code for a new not previously described eugenol hydroxylase which converts eugenol into coniferyl alcohol via

a quinone methide derivative by a process analogous to the reaction mechanism of p-cresol methyl hydroxylase. Another ORF of an unknown function was identified between the genes of the two subunits of eugenol hydroxylase. An ORF which was homologous to lignostilbene-a,b-dioxygenase was identified in a 5.0 kbp HindIII subfragment (H50). In addition one ORF was identified which was homologous to alcohol dehydrogenases. The structural gene vdh of ***vanillin*** dehydrogenase was identified in a 3.8 kbp HindIII/EcoRI subfragment. Upstream of this gene an ORF was localised which was homologous to enoyl-CoA hydratases produced by various organisms.
The complementing capacity of plasmid pE5-1 was attributed to the joint obtainment of the 1.2 and 1.8 kbp EcoRI fragments (E12 and E18). Fragment E 12 was completely, and fragment E 18 partially, sequenced. The structural gene cadh of coniferyl alcohol dehydrogenase, which contained an EcoRI cleavage site, was localised in these fragments. Using chromatographic methods the enzyme was isolated from the soluble fraction of the crude extract of cells of Pseudomonas sp. HR 199 grown on eugenol. An oligonucleotide sequence was deduced from the specific Nterminal amino acid sequence. A corresponding DNA probe hybridised with fragment E12, in which the region of the cadh gene encoding the N-terminus was localised.
A eugenol- and **ferulic** acid-negative mutant (mutant 6167) was complemented by obtaining a 9.4 kbp EcoRI fragment (E 94) of the hybrid cosmid pE5-1. A physical map of this fragment was prepared. The complementing property was localised in a 1.9 kbp EcoRI/HindIII subfragment. This fragment had incomplete ORF's (they extended beyond the EcoRI and HindIII cleavage sites) which were homologous to acetyl-CoA acetyl transferases of various organisms and to the "medium-chain acyl-CoA synthetase" produced by Pseudomonas oleovorans. Fragment E 94 was completely sequenced. Downstream of the aforementioned ORF's an ORF was located which was homologous to beta-ketothiolases. The structural gene of coniferylaldehyde dehydrogenase (caldh) was localised in a central position of fragment E 94. Using chromatographic methods the enzyme was isolated from the soluble fraction of the crude extract of cells of Pseudomonas sp. HR 199 grown on eugenol.
The conjugative transfer of hybrid cosmid pE207 into a large number of Pseudomonas strains resulted in the heterologous expression of the van A, van B and vdh genes and the eugenolhydroxylase genes in the transconjugants obtained. The obtainment of the plasmid of one strain allowed it to grow using eugenol as its carbon and energy source.
Growth conditions of the bacteria. Strains of Escherichia coli were grown at 37 degrees C. in a Luria-Bertani (LB) or M9 mineral medium (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Strains of Pseudomonas sp. and Alcaligenes eutrophus were grown at 30 degrees C. in a nutrient broth (NB, 0.8% by weight) or in a mineral medium (MM) (Schlegel, H. G. et al. 1961. Arch. Mikrobiol. 38: 209-222). **Ferulic** acid, **vanillin**, **vanillic** acid and protocatechuic acid were dissolved in dimethyl sulphoxide and added to the respective medium in a final concentration of 0.1% by weight. Eugenol was added to the medium directly in a final concentration of 0.1 vol.-%, or applied on filter paper (circular filters 595, Schleicher & Schuell, Dassel, Germany) to the lids of MM agar plates. For the growth of transconjugants of Pseudomonas sp., tetracyline and kanamycin were used in final concentrations of 25 mu g/ml and 300 mu g/ml, respectively.
Nitrosoguanidine mutagenesis. The nitrosoguanidine mutagenesis of Pseudomonas sp. HR 199 was carried out using a modified method according to Miller (Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Instead of the citrate buffer, a potassium phosphate (PP) buffer (100 mM, pH 7.0) was used. The final concentration of N-methyl-N'-nitro-N-nitrosoguanidine was 200 mu g/ml. The mutants obtained were screened with regard to the loss of their capacity to utilise eugenol, ***ferulic*** acid, **vanillin** and **vanillic** acid as growth substrates.
Qualitative and quantitative detection of metabolic intermediates in culture supernatants. Culture supernatants were analysed by high-pressure liquid chromatography (Knauer HPLC) either directly or after dilution with twice-distilled water. Chromatography was carried out on Nucleosil-100 C18 (7 mu m, 250 x 4 mm). The solvent used was 0.1 vol.-% formic acid and acetonitrile.
Purification of coniferyl alcohol dehydrogenase and coniferylaldehyde dehydrogenase. The purification processes were carried out at 4 degrees C.
Crude extract. Cells of Pseudomonas sp. HR 199 grown on eugenol were washed in a 10 mM sodium phosphate buffer with a pH of 7.5, resuspended in the same buffer and disrupted by being passed through a French press (Amicon, Silver

Spring, Md., USA) twice at a pressure of 1,000 psi. The cell homogenate was subjected to ultracentrifugation (1 h, 100,000 x g, 4 degrees C.), the soluble fraction of the crude extract being obtained as the supernatant.

DESCRIPTION OF FIGURES:

Anion exchange chromatography on DEAE Sephacel. The soluble fraction of the crude extract was dialysed overnight against a 10 mM sodium phosphate buffer with a pH of 7.5 containing 100 mM NaCl. The dialysate was applied to a DEAE Sephacel column (2. 6 cm x 35 cm, bed volumn (BV): 186 ml) equilibrated with a 10 mM sodium phosphate buffer of a pH of 7.5 containing 100 mM NaCl at a flow rate of 0.8 ml/min. The column was washed with two bed volumes of a 10 mM sodium phosphate buffer with a pH of 7.5 containing 100 mM NaCl. The elution of coniferyl alcohol dehydrogenase (CADH) and coniferylaldehyde dehydrogenase (CALDH) was carried out with a linear salt gradient of 100 to 500 mM NaCl in a 10 mM sodium phosphate buffer with a pH of 7.5 (2 x 150 ml). 5 ml fractions were collected. Fractions with high CADH and CALDH activities were combined in the corresponding DEAE pools respectively.

Gel filtration chromatography on Sephadex G200. The CADH DEAE pool was concentrated in a 50 ml Amicon ultrafiltration chamber via a Diaflo ultrafiltration membrane PM 30 (both from AMICON CORP., Lexington, USA) at a pressure of 290 kPa to a volume corresponding to approx. 2% of the Sephadex G200-BV. The concentrated protein solution was applied to a Sephadex G200 column (BV: 138 ml) equilibrated with a 10 mM sodium phosphate buffer with a pH of 7.5 containing 100 mM NaCl and eluted with the same buffer at a flow rate of 0.2 ml/min. 2 ml fractions were collected. Fractions with a high CADH activity were combined in the Sephadex G200 pool.

Hydrophobic interaction chromatography on butyl Sepharose 4B. The CADH Sephadex G200 pool was adjusted to 3 M NaCl and then applied to a butyl Sepharose 4B column (BV: 48 ml) equilibrated with a 10 mM sodium phosphate buffer with a pH of 7.5 containing 3 M NaCl (flow rate: 0.5 ml/min). The column was then washed with 2 BV of a 10 mM sodium phosphate buffer with a pH of 7.5 containing 3 M NaCl (flow rate: 1.0 ml/min). CADH was eluted with a linearly decreasing NaCl gradient of 3 to 0 M NaCl in a 10 mM sodium phosphate buffer with a pH of 7.5 (2 x 50 ml). 4 ml fractions were collected. Fractions with a high CADH activity were combined in the HIC pool and concentrated as described above.

Chromatography on hydroxyapatite. The CALDH DEAE pool was concentrated to 10 ml in a 50 ml Amicon ultrafiltration chamber via a Diaflo ultrafiltration membrane PM 30 (both from AMICON CORP., Lexington, USA) at a pressure of 290 kPa. The concentrated protein solution was applied to a hydroxyapatite column (BV: 80 ml) equilibrated with a buffer (10 mM NaCl in a 10 mM sodium phosphate buffer with a pH of 7.0) (flow rate: 2 ml/min). The column was then washed with 2.5 bed volumes of a buffer (flow rate: 2 ml/min). CALDH was eluted with a linearly increasing sodium phosphate gradient of 10 to 400 mM NaP (in each case containing 10 mM NaCL) (2 x 100 ml). 10 ml fractions were collected. Fractions with high CALDH activity were combined in the CALDH HA pool.

Gel filtration chromatography on Superdex HR 200 10/30. The CALDH HA pool was concentrated to 200 mu l (Amicon ultrafiltration chamber, ultrafiltration membrane PM 30) and applied to a Superdex HR 200 10/30 column (BV: 23.6 ml) equilibrated with a 10 mM sodium phosphate buffer with a pH of 7.0. CALDH was eluted with the same buffer at a flow rate of 0. 5 ml/min. 250 mu l fractions were collected. Fractions with high CALDH activity were combined in the CALDH Superdex pool.

Determination of coniferyl alcohol dehydrogenase activity. The CADH activity was determined at 30 degrees C. by means of an optical enzymatic test according to Jaeger et al. (Jaeger, E., L. Eggeling and H. Sahm. 1982. Current Microbiology. 6: 333-336) with the aid of a ZEISS PM 4 spectrophotometer fitted with a TE converter (both from ZEISS, Oberkochen, Germany) and a recorder. The reaction mixture with a volume of 1 ml contained 0.2 mmol of Tris/HCl (pH 9.0), 0.4 mu mol of coniferyl alcohol, 2 mu mol of AND, 0.1 mmol of semicarbazide and a solution of the enzyme ("Tris"=tris(hydroxymethyl)-aminomethane). The reduction of AND was monitored at 1=340 nm (e=6,3 cm2/ mu mol). The enzyme activity was recorded in units (U), 1 U corresponding to that quantity of enzyme which metabolises 1 mu mol of substrate per minute. The protein concentrations in the samples were determined according to the method described by Lowry et al. (Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. J. Biol. Chem. 193: 265-275).

Determination of the coniferylaldehyde dehydrogenase activity. The CALDH activity was determined at 30 degrees C. by an optical enzymatic test with the aid of a ZEISS PM 4 spectrophotometer fitted with a TE converter (both from ZEISS, Oberkochen, Germany) and a recorder. The reaction mixture of a volume of1 ml contained a 10 mM Tris/HCl buffer (pH 8.8), 5.6 mM coniferylaldehyde, 3 mM AND and a solution of the enzyme. The oxidation of coniferylaldehyde to form

ferulic acid was monitored at 1=400 nm (e=34 cm2/ mu mol). The enzyme activity was recorded in units (U), 1 U corresponding to that quantity of enzyme which metabolises 1 mu mol of substrate per minute. The protein concentration in the samples was determined according to the method described by Lowry et al. (Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. J. Biol. Chem. 193: 265-275).
Electrophoretic methods. The separation of protein-containing extracts was carried out in 7.4% by weight polyacrylamide gels under native conditions according to the method described by Stegemann et al. (Stegemann et al. 1973. Z. Naturforsch. 28c: 722-732) and under denaturing conditions in 11.5% by weight polyacrylamide gels according to the method described by Laemmli (Laemmli, U. K. 1970. Nature (London) 227: 680-685). Serva Blue R was used for non-specific protein staining. For specifically staining coniferyl alcohol, coniferylaldehyde and **vanillin** dehydrogenase the gels were placed for 20 mins in a new 100 mM PP buffer (pH 7.0) and then incubated at 30 degrees C. in the same buffer, to which 0.08% by weight of AND, 0.04% by weight of p-nitroblue-tetrazolium chloride, 0.003% by weight of phenazine methosulphate and 1 mM of the respective substrate had been added, until the corresponding coloured bands appeared.
The transfer of proteins from polyacrylamide gels to PVDF membranes. Proteins were transferred from SDS polyacrylamide gels to PVDF membranes (Waters-Milipore, Bedford, Mass., USA) with the aid of a semidry fast blot device (B32/33 from Biometra, Gottingen, Germany) according to the manufacturer's instructions.
Determination of N-terminal amino acid sequences. The determination of N-terminal amino acid sequences was carried out with the aid of a protein peptide sequencer (of type 477 A, Applied Biosystems, Foster City, USA) and a PTH analyser, according to the manufacturer's instructions.
Isolation and manipulation of DNA. The isolation of genomic DNA was carried out by the method described by Marmur (Marmur, J. 1961. Mol. Biol. 3: 208-218). Megaplasmid DNA was isolated according to the method described by Nies et al. (Nies, D., et al. 1987. J. Bacteriol. 169: 4865-4848). The isolation and analysis of other plasmid DNA or DNA restriction fragments, the packaging of hybrid cosmids in 1-phage particles and the transduction of E. coli. was carried out by standard methods (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).
Transfer of DNA. The preparation and transformation of competent Escherichia coli cells was carried out by the method described by Hanahan (Hanahan, D. 1983. J. Mol. Biol. 166: 557-580). Conjugative plasmid transfer between plasmid-containing Escherichia coli S17-1 strains (donor) and Pseudomonas sp. strains (recipient) and Alcaligenes eutrophus (recipient) was carried out on NB agar plates according to the method described by Friedrich et al. (Friedrich, B. et al. 1981. J. Bacteriol. 147: 198-205) or by a "minicomplementation method" on MM agar plates using 0.5% by weight of gluconate as the carbon source and 25 mu g/ml of tetracylin or 300 mu g/ml of kanamycin. In this process cells of the recipient were applied in one direction in the form of an inoculation line. After 5 minutes cells of the donor strains were then applied in the form of inoculation lines crossing the recipient inoculation line. After incubation for 48 h at 30 degrees C. the transconjugants grew directly downstream of the crossing point, whereas neither the donor nor the recipient strain was capable of growth.
Hybridisation experiments. DNA restriction fragments were electrophoretically separated in an 0.8% by weight agarose gel in a 50 mM Tris, 50 mM boric acid and 1.25 mM EDTA buffer (pH 8. 5) (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The transfer of the denatured DNA from the gel to a positively charged nylon membrane (pore size: 0.45 mu m, Pall Filtrationstechnik, Dreieich, Germany), the subsequent hybridisation with biotinylated or 32P-labelled DNA probes and the production of these DNA probes was carried out according to standard methods (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

DESCRIPTION OF FIGURES:

The synthesis of oligonucleotides. Using desoxynucleoside phosphoramidites as the starting material, oligonucleotides were synthesised on a 0.2 mu mol scale (Beaucage, S. L., and M. H. Caruthers. 1981. Tetrahedron Lett. 22: 1859-1862). The synthesis was carried out in a Gene Assembler Plus according to the manufacturer's instructions (Pharmacia-LKB, Uppsala, Sweden) . The elimination of the protecting groups was carried out by incubation for 15 h at 55 degrees C. in a 25 vol.-% aqueous ammonia solution. The oligonucleotides were finally

purified in an NAP-5 column (Pharmacia-LKB, Uppsala, Sweden).
DNA sequencing. The determination of nucleotide sequences was carried out by the didesoxy chain termination method described by Sanger et al. (Sanger et al. 1977. Proc. Natl. Acad. Sci. USA 74: 5463-5467) using (alpha-35S)dATP and a T7 polymerase sequencing kit (Pharmacia-LKB). 7-Deazaguanosine-5'triphosphate was used instead of dGTP (Mizusawa, S. et al. 1986. Nucleic Acids Res.14: 1319-1324). The products of the sequencing reactions were separated in a 6% by weight polyacrylamide gel in a 100 mM Tris/HCl, 83 mM boric acid and 1 mM EDTA buffer (pH 8.3) containing 42% by weight urea, an S2 sequencing apparatus (GIBCO/BRL, Bethesda Research Laboratories GmbH, Eggenstein, Germany) being used according to the manufacturer's instructions. After electrophoresis the gels were incubated for 30 mins in 10 vol.-% acetic acid and, after washing briefly in water, dried for 2 hours at 80 degrees C. Kodak X-OMAT AR X-ray films (Eastman Kodak Company, Rochester, N.Y., USA) were used for the autoradiography of the dried gels. In addition DNA sequences were also determined "nonradioactively" with the aid of an "LI-COR DNA Sequencer Model 4000L" (LI-COR Inc., Biotechnology Division, Lincoln, Neb., USA) using a "Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Life Science, Amersham International plc, Little Chalfont, Buckinghamshire, England), in each case according to the manufacturer's instructions.
Various sequencing strategies were used: With the aid of synthetic oligonucleotides sequencing was carried out by the "Primer-hopping Strategy" described by Strauss et al. (Strauss, E. C. et al. 1986. Anal. Biochem. 154: 353-360). If only "universal" and "reverse primers" were used hybrid plasmids were used as "template DNA", the inserted DNA fragments of which had been unidirectionally shortened with the aid of an "Exo III/Mung Bean Nuclease Deletion" kit (Stratagene Cloning Systems, La Jolla, Calif., USA) according to the manufacturer's instructions.
Chemicals, biochemicals and enzymes: Restriction enzymes, T4 DNA ligase, lambda DNA and enzymes and substrates for the optical enzymatic tests were obtained from C. F. Boehringer & Sohne (Mannheim, Germany) or from GIBCO/BRL (Eggenstein, Germany). (a35S)dATP and (g-32P)ATP were obtained from Amersham/Buchler (Braunschweig, Germany). NA-type agarose was obtained from Pharmacia-LKB (Uppsala, Sweden). All the other chemicals were from Haarmann & Reimer (Holzminden, Germany), E. Merck AG (Darmstadt, Germany), Fluka Chemic (Buchs, Switzerland), Serva Feinbiochemica (Heidelberg, Germany) or Sigma Chemie (Deisenhofen, Germany).!

AB The present invention relates to synthetic enzymes for the production of coniferyl alcohol, coniferylaldehyde, ferulic acid, **vanillin** and **vanillic** acid, the use thereof for the production of coniferyl alcohol, coniferylaldehyde, ferulic acid, **vanillin** and **vanillic** acid, DNA coding for these enzymes and microorganisms transformed with this DNA.

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GI 1 Drawing Sheet(s), 1 Figure(s).

FIG. 1 is a physical map of cloned fragments, subfragments, and sequenced areas.

The present invention relates to synthetic enzymes for the production of coniferyl alcohol, coniferylaldehyde, **ferulic** acid, **vanillin** and **vanillic** acid from eugenol.

Synthetic enzymes according to the invention are for example:

a) eugenol hydroxylase,
b) coniferyl alcohol dehydrogenase,
c) coniferylaldehyde dehydrogenase,
d) **ferulic** acid **deacylase** and
e) **vanillin** dehydrogenase.

The invention also relates to DNA coding for the abovementioned enzymes and cosmid clones containing this DNA as well as vectors containing this DNA and microorganisms transformed with the DNA or the vectors. It also relates to the use of the DNA for the transformation of microorganisms for the production of coniferyl alcohol, coniferylaldehyde, **ferulic** acid, **vanillin** and **vanillic** acid. The invention also relates to partial sequences of the DNA and functional equivalents. Functional equivalents are understood to be those derivatives in which individual nucleobases have been substituted (wobble substitutions) without resulting in any functional changes. In relation to proteins, amino acids can also be substituted without resulting in any functional changes.

The invention also relates to the individual steps for the production of

coniferyl alcohol, coniferylaldehyde, **ferulic** acid, **vanillin** and **vanillic** acid from eugenol, i.e. in concrete terms:

a) the process for the production of coniferyl alcohol from eugenol carried out in the presence of eugenol hydroxylase;
b) the process for the production of coniferylaldehyde from coniferyl alcohol carried out in the presence of coniferyl alcohol dehydrogenase;
c) the process for the production of **ferulic** acid from coniferylaldehyde carried out in the presence of coniferylaldehyde dehydrogenase;
d) the process for the production of **vanillin** from **ferulic** acid carried out in the presence of **ferulic** acid **deacylase**;
e) the process for the production of **vanillic** acid from **vanillin** carried out in the presence of **vanillin** dehydrogenase.

After NMG mutagenesis mutants with defects in individual stages of the catabolism of eugenol were obtained from the eugenolutilising Pseudomonas sp. strain HR 199 (DSM 7063). Using total DNA of wild-type Pseudomonas sp. HR 199 partially digested with EcoRI a gene library was constructed in the pVK100 cosmid, which has a broad host spectrum and can also be replicated in stable form in pseudomonads. After packaging in 1-phage particles the hybrid cosmids were transduced to E. coli S17-1. The gene library comprised 1330 recombinant E. coli S17-1 clones. The hybrid cosmid of each clone was transferred by conjugation into two eugenol-negative mutants (mutants 6164 and 6165) of the Pseudomonas sp. HR 199 strain and tested for a possible capacity for complementation. In this test two hybrid cosmids (pE207 and pE115) were identified, the obtainment of which restored mutant 6165's capacity to utilise eugenol. One hybrid cosmid (pE5-1) resulted in the complementation of mutant 6164.

The complementing capacity of plasmids pE207 and pE115 was attributed to a 23 kbp EcoRI fragment (E230). A physical map of this fragment was prepared and the fragment completely sequenced. The genes vanA and vanB which code for **vanillate** demethylase were localised in a 11.2 kbp HindIII subfragment (H110).

Another open reading frame (ORF) was found to be homologous to gglutamyl cysteine synthetase produced by Escherichia coli. An additional ORF, which was homologous to formaldehyde dehydrogenases, was identified between the aforementioned ORF and the vanB gene. Two additional ORF's were found to be homologous to the cytochrome C subunit or the flavoprotein subunit of p-cresol methylhydroxylase, respectively produced by Pseudomonas putida. In the Pseudomonas sp. HR 199 strain, these ORF's code for a new not previously described eugenol hydroxylase which converts eugenol into coniferyl alcohol via a quinone methide derivative by a process analogous to the reaction mechanism of p-cresol methyl hydroxylase. Another ORF of an unknown function was identified between the genes of the two subunits of eugenol hydroxylase. An ORF which was homologous to lignostilbene-a,b-dioxygenase was identified in a 5.0 kbp HindIII subfragment (H50). In addition one ORF was identified which was homologous to alcohol dehydrogenases. The structural gene vdh of **vanillin** dehydrogenase was identified in a 3.8 kbp HindIII/EcoRI subfragment. Upstream of this gene an ORF was localised which was homologous to enoyl-CoA hydratases produced by various organisms.

The complementing capacity of plasmid pE5-1 was attributed to the joint obtainment of the 1.2 and 1.8 kbp EcoRI fragments (E12 and E18). Fragment E 12 was completely, and fragment E 18 partially, sequenced. The structural gene cadh of coniferyl alcohol dehydrogenase, which contained an EcoRI cleavage site, was localised in these fragments. Using chromatographic methods the enzyme was isolated from the soluble fraction of the crude extract of cells of Pseudomonas sp. HR 199 grown on eugenol. An oligonucleotide sequence was deduced from the specific Nterminal amino acid sequence. A corresponding DNA probe hybridised with fragment E12, in which the region of the cadh gene encoding the N-terminus was localised.

A eugenol- and **ferulic** acid-negative mutant (mutant 6167) was complemented by obtaining a 9.4 kbp EcoRI fragment (E 94) of the hybrid cosmid pE5-1. A physical map of this fragment was prepared. The complementing property was localised in a 1.9 kbp EcoRI/HindIII subfragment. This fragment had incomplete ORF's (they extended beyond the EcoRI and HindIII cleavage sites) which were homologous to acetyl-CoA acetyl transferases of various organisms and to the "medium-chain acyl-CoA synthetase" produced by Pseudomonas oleovorans. Fragment E 94

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Growth conditions of the bacteria. Strains of Escherichia coli were grown at 37 degrees C. in a Luria-Bertani (LB) or M9 mineral medium (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Strains of Pseudomonas sp. and Alcaligenes eutrophus were grown at 30 degrees C. in a nutrient broth (NB, 0.8% by weight) or in a mineral medium (MM) (Schlegel, H. G. et al. 1961. Arch. Mikrobiol. 38: 209-222). **Ferulic** acid, **vanillin**, **vanillic** acid and protocatechuic acid were dissolved in dimethyl sulphoxide and added to the respective medium in a final concentration of 0.1% by weight. Eugenol was added to the medium directly in a final concentration of 0.1 vol.-%, or applied on filter paper (circular filters 595, Schleicher & Schuell, Dassel, Germany) to the lids of MM agar plates. For the growth of transconjugants of Pseudomonas sp., tetracyline and kanamycin were used in final concentrations of 25 mu g/ml and 300 mu g/ml, respectively.

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Crude extract. Cells of Pseudomonas sp. HR 199 grown on eugenol were washed in a 10 mM sodium phosphate buffer with a pH of 7.5, resuspended in the same buffer and disrupted by being passed through a French press (Amicon, Silver Spring, Md., USA) twice at a pressure of 1,000 psi. The cell homogenate was subjected to ultracentrifugation (1 h, 100,000 x g, 4 degrees C.), the soluble fraction of the crude extract being obtained as the supernatant.

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Gel filtration chromatography on Superdex HR 200 10/30. The CALDH HA pool was concentrated to 200 mu l (Amicon ultrafiltration chamber, ultrafiltration membrane PM 30) and applied to a Superdex HR 200 10/30 column (BV: 23.6 ml) equilibrated with a 10 mM sodium phosphate buffer with a pH of 7.0. CALDH was eluted with the same buffer at a flow rate of 0. 5 ml/min. 250 mu 1 fractions were collected. Fractions with high CALDH activity were combined in the CALDH Superdex pool.

Determination of coniferyl alcohol dehydrogenase activity. The CADH activity was determined at 30 degrees C. by means of an optical enzymatic test according to Jaeger et al. (Jaeger, E., L. Eggeling and H. Sahm. 1982. Current Microbiology. 6: 333-336) with the aid of a ZEISS PM 4 spectrophotometer fitted with a TE converter (both from ZEISS, Oberkochen, Germany) and a recorder. The reaction mixture with a volume of 1 ml contained 0.2 mmol of Tris/HCl (pH 9.0), 0.4 mu mol of coniferyl alcohol, 2 mu mol of AND, 0.1 mmol of semicarbazide and a solution of the enzyme ("Tris"=tris(hydroxymethyl)-aminomethane). The reduction of AND was monitored at 1=340 nm (e=6,3 cm2/ mu mol). The enzyme activity was recorded in units (U), 1 U corresponding to that quantity of enzyme which metabolises 1 mu mol of substrate per minute. The protein concentrations in the samples were determined according to the method described by Lowry et al. (Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. J. Biol. Chem. 193: 265-275).

Determination of the coniferylaldehyde dehydrogenase activity. The CALDH activity was determined at 30 degrees C. by an optical enzymatic test with the aid of a ZEISS PM 4 spectrophotometer fitted with a TE converter (both from ZEISS, Oberkochen, Germany) and a recorder. The reaction mixture of a volume of1 ml contained a 10 mM Tris/HCl buffer (pH 8.8), 5.6 mM coniferylaldehyde, 3 mM AND and a solution of the enzyme. The oxidation of coniferylaldehyde to form ferulic acid was monitored at 1=400 nm (e=34 cm2/ mu mol). The enzyme activity was recorded in units (U), 1 U corresponding to that quantity of enzyme which metabolises 1 mu mol of substrate per minute. The protein concentration in the samples was determined according to the method described by Lowry et al. (Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. J. Biol. Chem. 193: 265-275).

Electrophoretic methods. The separation of protein-containing extracts was carried out in 7.4% by weight polyacrylamide gels under native conditions according to the method described by Stegemann et al. (Stegemann et al. 1973. Z. Naturforsch. 28c: 722-732) and under denaturing conditions in 11.5% by weight polyacrylamide gels according to the method described by Laemmli (Laemmli, U. K. 1970. Nature (London) 227: 680-685). Serva Blue R was used for non-specific protein staining. For specifically staining coniferyl alcohol, coniferylaldehyde and **vanillin** dehydrogenase the gels were placed for 20 mins in a new 100 mM PP buffer (pH 7.0) and then incubated at 30 degrees C. in the same buffer, to which 0.08% by weight of AND, 0.04% by weight of p-nitroblue-tetrazolium chloride,

0.003% by weight of phenazine methosulphate and 1 mM of the respective substrate had been added, until the corresponding coloured bands appeared.

The transfer of proteins from polyacrylamide gels to PVDF membranes. Proteins were transferred from SDS polyacrylamide gels to PVDF membranes (Waters-Milipore, Bedford, Mass., USA) with the aid of a semidry fast blot device (B32/33 from Biometra, Gottingen, Germany) according to the manufacturer's instructions.

Determination of N-terminal amino acid sequences. The determination of N-terminal amino acid sequences was carried out with the aid of a protein peptide sequencer (of type 477 A, Applied Biosystems, Foster City, USA) and a PTH analyser, according to the manufacturer's instructions.

Isolation and manipulation of DNA. The isolation of genomic DNA was carried out by the method described by Marmur (Marmur, J. 1961. Mol. Biol. 3: 208-218). Megaplasmid DNA was isolated according to the method described by Nies et al. (Nies, D., et al. 1987. J. Bacteriol. 169: 4865-4848). The isolation and analysis of other plasmid DNA or DNA restriction fragments, the packaging of hybrid cosmids in 1-phage particles and the transduction of E. coli. was carried out by standard methods (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Transfer of DNA. The preparation and transformation of competent Escherichia coli cells was carried out by the method described by Hanahan (Hanahan, D. 1983. J. Mol. Biol. 166: 557-580). Conjugative plasmid transfer between plasmid-containing Escherichia coli S17-1 strains (donor) and Pseudomonas sp. strains (recipient) and Alcaligenes eutrophus (recipient) was carried out on NB agar plates according to the method described by Friedrich et al. (Friedrich, B. et al. 1981. J. Bacteriol. 147: 198-205) or by a "minicomplementation method" on MM agar plates using 0.5% by weight of gluconate as the carbon source and 25 mu g/ml of tetracylin or 300 mu g/ml of kanamycin. In this process cells of the recipient were applied in one direction in the form of an inoculation line. After 5 minutes cells of the donor strains were then applied in the form of inoculation lines crossing the recipient inoculation line. After incubation for 48 h at 30 degrees C. the transconjugants grew directly downstream of the crossing point, whereas neither the donor nor the recipient strain was capable of growth.

Hybridisation experiments. DNA restriction fragments were electrophoretically separated in an 0.8% by weight agarose gel in a 50 mM Tris, 50 mM boric acid and 1.25 mM EDTA buffer (pH 8. 5) (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The transfer of the denatured DNA from the gel to a positively charged nylon membrane (pore size: 0.45 mu m, Pall Filtrationstechnik, Dreieich, Germany), the subsequent hybridisation with biotinylated or 32P-labelled DNA probes and the production of these DNA probes was carried out according to standard methods (Sambrook, J. E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

The synthesis of oligonucleotides. Using desoxynucleoside phosphoramidites as the starting material, oligonucleotides were synthesised on a 0.2 mu mol scale (Beaucage, S. L., and M. H. Caruthers. 1981. Tetrahedron Lett. 22: 1859-1862). The synthesis was carried out in a Gene Assembler Plus according to the manufacturer's instructions (Pharmacia-LKB, Uppsala, Sweden) . The elimination of the protecting groups was carried out by incubation for 15 h at 55 degrees C. in a 25 vol.-% aqueous ammonia solution. The oligonucleotides were finally purified in an NAP-5 column (Pharmacia-LKB, Uppsala, Sweden).

DNA sequencing. The determination of nucleotide sequences was carried out by the didesoxy chain termination method described by Sanger et al. (Sanger et al. 1977. Proc. Natl. Acad. Sci. USA 74: 5463-5467) using (alpha-35S)dATP and a T7 polymerase sequencing kit (Pharmacia-LKB). 7-Deazaguanosine-5'triphosphate was used instead of dGTP (Mizusawa, S. et al. 1986. Nucleic Acids Res.14: 1319-1324). The products of the sequencing reactions were separated in a 6% by weight polyacrylamide gel in a 100 mM Tris/HCl, 83 mM boric acid and 1 mM EDTA buffer (pH 8.3) containing 42% by weight urea, an S2 sequencing apparatus (GIBCO/BRL, Bethesda Research Laboratories GmbH, Eggenstein, Germany) being used according to the manufacturer's instructions. After electrophoresis the gels were incubated for 30 mins in 10 vol.-% acetic acid and, after

washing briefly in water, dried for 2 hours at 80 degrees C. Kodak X-OMAT AR X-ray films (Eastman Kodak Company, Rochester, N.Y., USA) were used for the autoradiography of the dried gels. In addition DNA sequences were also determined "nonradioactively" with the aid of an "LI-COR DNA Sequencer Model 4000L" (LI-COR Inc., Biotechnology Division, Lincoln, Neb., USA) using a "Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Life Science, Amersham International plc, Little Chalfont, Buckinghamshire, England), in each case according to the manufacturer's instructions.

Various sequencing strategies were used: With the aid of synthetic oligonucleotides sequencing was carried out by the "Primer-hopping Strategy" described by Strauss et al. (Strauss, E. C. et al. 1986. Anal. Biochem. 154: 353-360). If only "universal" and "reverse primers" were used hybrid plasmids were used as "template DNA", the inserted DNA fragments of which had been unidirectionally shortened with the aid of an "Exo III/Mung Bean Nuclease Deletion" kit (Stratagene Cloning Systems, La Jolla, Calif., USA) according to the manufacturer's instructions.

Chemicals, biochemicals and enzymes: Restriction enzymes, T4 DNA ligase, lambda DNA and enzymes and substrates for the optical enzymatic tests were obtained from C. F. Boehringer & Sohne (Mannheim, Germany) or from GIBCO/BRL (Eggenstein, Germany). (a35S)dATP and (g-32P)ATP were obtained from Amersham/Buchler (Braunschweig, Germany). NA-type agarose was obtained from Pharmacia-LKB (Uppsala, Sweden). All the other chemicals were from Haarmann & Reimer (Holzminden, Germany), E. Merck AG (Darmstadt, Germany), Fluka Chemic (Buchs, Switzerland), Serva Feinbiochemica (Heidelberg, Germany) or Sigma Chemie (Deisenhofen, Germany).!

L4 ANSWER 3 OF 6 IFIPAT COPYRIGHT 2004 IFI on STN DUPLICATE 2

AN 10238990 IFIPAT;IFIUDB;IFICDB

TITLE: SYNTHETIC ENZYMES FOR THE PRODUCTION OF CONIFERYL ALCOHOL, CONIFERYLALDEHYDE, FERULIC ACID, **VANILLIN** AND **VANILLIC** ACID AND THEIR USE

INVENTOR(S): PRIEFERT; HORST, TELGTE, DE
RABENHORST; JURGEN, HOXTER, DE
STEINBUCHEL; ALEXANDER, ALTENBERGE, DE

PATENT ASSIGNEE(S): Unassigned

PATENT ASSIGNEE PROBABLE: Haarmann & Reimer GmbH DE (Probable)

AGENT: Norris Mc Laughlin & Marcus, P.A., 220 East 42nd Street, 30th floor, New York, NY 10017, US

	NUMBER	PK	DATE
PATENT INFORMATION:	US 2002182697	A1	20021205
APPLICATION INFORMATION:	US 1997-976063		19971121

	NUMBER	DATE
PRIORITY APPLN. INFO.:	DE 1996-19649655	19961129
FAMILY INFORMATION:	US 2002182697	20021205
	US 6524831	20030225

DOCUMENT TYPE: Utility
Patent Application - First Publication

FILE SEGMENT: CHEMICAL
APPLICATION

NUMBER OF CLAIMS: 13

AB The present invention relates to synthetic enzymes for the production of coniferyl alcohol, coniferylaldehyde, ferulic acid, **vanillin** and **vanillic** acid, the use thereof for the production of coniferyl alcohol, coniferylaldehyde, ferulic acid, **vanillin** and **vanillic** acid, DNA coding for these enzymes and microorganisms transformed with this DNA.

CLMN 13

L4 ANSWER 4 OF 6 USPATFULL on STN

ACCESSION NUMBER: 2001:231389 USPATFULL

TITLE: Process for the preparation of aromatic carbonyl compounds from styrenes

INVENTOR(S): Gatfield, Ian-Lucas, Hoxter, Germany, Federal Republic of

Hilmer, Jens-Michael, Hoxter, Germany, Federal Republic of

PATENT ASSIGNEE(S): Haarmann & Reimer GmbH, Holzminden, Germany, Federal Republic of (non-U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 6331655	B1	20011218	
APPLICATION INFO.:	US 2000-591637		20000609	(9)

	NUMBER	DATE
PRIORITY INFORMATION:	DE 1999-19928158	19990619

DOCUMENT TYPE: Utility
FILE SEGMENT: GRANTED
PRIMARY EXAMINER: Killos, Paul J.
ASSISTANT EXAMINER: Chaudhry, Mahreen
LEGAL REPRESENTATIVE: Gil, Joseph C., Cheung, Noland J.
NUMBER OF CLAIMS: 13
EXEMPLARY CLAIM: 1
LINE COUNT: 382
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention relates to a novel process for the preparation of aromatic carbonyl compounds by oxidative cleavage of styrenes using lipases and hydrogen peroxide or hydrogen peroxide donors in the presence of carboxylic acids or carboxylic esters.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L4 ANSWER 5 OF 6 BIOTECHDS COPYRIGHT 2004 THOMSON DERWENT/ISI on STN
ACCESSION NUMBER: 1998-07808 BIOTECHDS
TITLE: New enzyme of eugenol catabolism, DNA, cosmid clones, vectors and transformed microorganisms;
coniferyl alcohol, coniferyl aldehyde, ferulic acid, **vanillin** and **vanillic** acid production
via enzyme-catalyzed eugenol catabolism; cosmid clone and vector plasmid pVK100 expression in Pseudomonas sp.
AUTHOR: Steinbuechel A; Priefert H; Rabenhorst J
PATENT ASSIGNEE: Haarmann+Reimer
LOCATION: Holzminden, Germany.
PATENT INFO: EP 845532 3 Jun 1998
PRIORITY INFO: DE 1996-1064965 29 Nov 1996
DOCUMENT TYPE: Patent
LANGUAGE: German
OTHER SOURCE: WPI: 1998-288749 [26]
AN 1998-07808 BIOTECHDS

AB Enzymes for the synthesis of coniferyl alcohol, coniferyl aldehyde, **ferulic** acid, **vanillin** and **vanillic** acid from eugenol are new. Also claimed are: DNA (I) coding for the enzymes; cosmid clones and vectors containing (I); and microorganisms transformed with (I). The microorganisms are used to produce the above compounds. The enzymes are used for the conversion of eugenol to coniferyl alcohol in the presence of eugenol-hydroxylase, conversion of coniferyl alcohol to coniferyl aldehyde in the presence of coniferyl alcohol-dehydrogenase, conversion of coniferyl aldehyde to **ferulic** acid in the presence of coniferyl aldehyde-dehydrogenase, conversion of **ferulic** acid to **vanillin** in the presence of **ferulic** acid **deacylase** and for the conversion of **vanillin** to **vanillic** acid in the presence of **vanillin**-dehydrogenase. Examples relate to generation of Pseudomonas sp. HR199 mutants with defective eugenol catabolism, construction of a HR199 genomic library in cosmid vector plasmid pVK100, identifying hybrid cosmids capable of complementing the defective eugenol catabolism of the mutants, analysis of a 23 kb EcoRI fragment of pE207 and further characterization. (108pp)

L4 ANSWER 6 OF 6 CAPLUS COPYRIGHT 2004 ACS on STN
ACCESSION NUMBER: 1998:379165 CAPLUS
DOCUMENT NUMBER: 129:53451
TITLE: Eugenol-catabolizing enzymes, recombinant microbes expressing these enzymes, and production of coniferyl

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                             alcohol, coniferyl aldehyde, ferulic acid,
                             vanillin, vanillic acid with said
                             enzymes
INVENTOR(S):                 Steinbuechel, Alexander; Priefert, Horst; Rabenhorst,
                             Juergen
PATENT ASSIGNEE(S):          Haarmann und Reimer G.m.b.H., Germany
SOURCE:                      Ger. Offen., 26 pp.
                             CODEN: GWXXBX
DOCUMENT TYPE:               Patent
LANGUAGE:                    German
FAMILY ACC. NUM. COUNT:      1
PATENT INFORMATION:

     PATENT NO.       KIND  DATE          APPLICATION NO.  DATE
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     DE 19649655       A1   19980604      DE 1996-19649655 19961129
     EP 845532         A2   19980603      EP 1997-120058   19971117
     EP 845532         A3   20000105
         R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
             IE, FI
     US 2002182697     A1   20021205      US 1997-976063   19971121
     US 6524831        B2   20030225
     JP 10155496       A2   19980616      JP 1997-343875   19971128
     US 2003228670     A1   20031211      US 2000-750986   20001228
PRIORITY APPLN. INFO.:                 DE 1996-19649655 A  19961129
                                       US 1997-976063   A3 19971121
AB   Eugenol-catabolizing enzymes eugenol hydroxylase, coniferyl alc.
     dehydrogenase, coniferyl aldehyde dehydrogenase, ferulic acid
     deacylase and vanillin dehydrogenase; microbes
     expressing genes for these enzymes; use of the recombinant microbes for
     prodn. of coniferyl alc., coniferyl aldehyde, ferulic acid,
     vanillin and vanillic acid; and methods for prodn. of
     these compds. using the enzymes are disclosed.  Genes for the enzymes
     mentioned were cloned from Pseudomonas HR 199 and expressed in other
     Pseudomonas species and in Alcaligenes eutrophus.


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     (FILE 'HOME' ENTERED AT 16:49:17 ON 16 JUN 2004)

     INDEX 'ADISCTI, ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, AQUASCI,
     BIOBUSINESS, BIOCOMMERCE, BIOSIS, BIOTECHABS, BIOTECHDS, BIOTECHNO, CABA,
     CANCERLIT, CAPLUS, CEABA-VTB, CEN, CIN, CONFSCI, CROPB, CROPU, DISSABS,
     DDFB, DDFU, DGENE, DRUGB, DRUGMONOG2, ...' ENTERED AT 16:49:40 ON 16 JUN
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               1   FILE CAPLUS
               0*  FILE FEDRIP
               3   FILE IFIPAT
               3   FILE USPATFULL
               1   FILE USPAT2
               1   FILE WPIDS
               1   FILE WPINDEX
L1              QUE DEACYLAS? (S) VANILL? (S) FERU?
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                SEA (DEACYLAS?(S)FERU?) AND VANILL?
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               1   FILE BIOTECHABS
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FILE 'IFIPAT, USPATFULL, BIOTECHDS, CAPLUS, USPAT2, WPIDS' ENTERED AT 16:52:16 ON 16 JUN 2004

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L3            10 S (DEACYLAS?(S)FERU?) AND VANILL?
L4             6 DUP REM L3 (4 DUPLICATES REMOVED)

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FULL ESTIMATED COST                                     59.43      61.92

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